Protein prenylcysteine analog inhibits agonist-receptor-mediated signal transduction in human platelets

(panreylated proteins/carboxyl methylation/GTP-binding proteins)

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ABSTRACT  Signal transduction components, including the Ras superfamily of low molecular weight GTP-binding proteins and the γ subunits of heterotrimeric G proteins, are reversibly carboxyl methylated at C-terminal prenylcysteine residues. We have previously shown that the prenylcysteine analog N-acetyl-S-trans,trans-farnesyl-L-cysteine (AFC) inhibits its carboxyl methylation of these proteins in human platelets. Here we show that concentrations of AFC that inhibit Ras carboxyl methylation (10–50 μM) also block responses to agonists such as ADP, collagen, arachidonic acid, U46619 (a stable analog of prostaglandin H2), thrombin, and guanosine 5′-[γ-thio]triphosphate. AFC does not inhibit aggregation induced by effectors such as ionomycin, phorbol 12,13-diibutyrate, and bacterial phospholipase C that bypass G proteins to activate platelets at the level of cytosolic Ca2+ concentration and protein kinase C. These findings indicate that AFC inhibits agonist-receptor-mediated signal transduction in human platelets.

The finding that Ras proteins and the heterotrimeric G proteins are methylated at the carboxyl group of the C terminus has raised the possibility that this type of reversible covalent modification may play a role in signal transduction (1, 2). These GTP-binding proteins function to relay information from membrane receptors to control the cytoplasmic levels of second messengers, such as 3′,5′-AMP, cyclic AMP (cAMP), cytotoxic Caa2+ (3, 4), and diacylglycerol (DAG) (3, 4), that act in turn to regulate protein kinases. Ras proteins and the γ subunits of G proteins are initially synthesized with a characteristic C-terminal sequence, CXXX (a cysteine followed by three, generally aliphatic, residues) (2, 5). CXXX sequences provide a recognition site for cytosolic prenyltransferase enzymes, which catalyze the attachment of farnesyl or geranylgeranyl polyisoprenoid groups to other linkage to the cysteine (2, 6). The three residues distal to the cysteine in prenylated CXXX sequences are removed by membrane-associated protease activities (7), and the exposed prenylcysteine α-carboxylate undergoes methylation by a membrane-bound S-adenosylmethionine:prenylcysteine methyltransferase (8, 9).

Results with inhibitors of polyprenyl synthesis, such as compactin and mevinolin (lovastatin), indicate that prenylation is essential for the function of Ras proteins and γ subunits of the heterotrimeric G proteins (10–13). Prenylation is required for all subsequent steps in CXXX processing (13, 14), and without prenylation, these proteins cannot localize to their sites of action at the plasma membrane (15–17). Classical heterotrimeric G proteins as well as low molecular weight G proteins such as Rap1a, Rap1b, Rap2, and Rap2b are present in human platelets (18–27). To specifically investigate the role of carboxyl methylation in G-protein-dependent signal transduction, we have examined the effects of a prenylcysteine methyltransferase inhibitor, N-acetyl-S-trans,trans-farnesyl-L-cysteine (AFC), on agonist-receptor-mediated signal transduction in human platelets. AFC has previously been shown to be an inhibitor of protein carboxyl methylation in platelets (28) and other cell types (9, 29, 30). Here we show that AFC but not N-acetyl-S-trans,farnesyl-L-cysteine (AGC), which is a poor inhibitor of protein methylation (9), inhibits agonist-induced platelet activation. AFC appears to function by blocking agonist-receptor–G-protein-mediated stimulus–response coupling between receptors and second messengers. AFC does not inhibit platelet responses induced by agents such as ionomycin, phospholipase C (PLC), and phorbol esters that bypass G proteins and directly activate second messenger-dependent signal-transduction pathways.

MATERIALS AND METHODS

Materials. AFC was prepared and was characterized by NMR, mass spectrometry, and HPLC as described previously (31). 5-Hydroxy-[14C]tryptamine creatinine sulfate (14C]serotonin, 57 μCi/mmol; 1 Ci = 37 GBq) was obtained from Amersham. ADP, thrombin, arachidonic acid, U46619 (a stable analog of prostaglandin H2), acetylsalicylic acid (aspirin), saponin, and bacterial PLC (from Clostridium perfringens) were purchased from Sigma. Collagen was obtained from Worthington. Ionomycin, guanosine 5′-[γ-thio]triphosphate (GTP[yS]), and fura 2/AM were supplied by Calbiochem. Phorbol 12,13-dibutyrate (PDBu) was obtained from LC Services (Woburn, MA). Staurosporine was purchased from Seikagaku America (St. Petersburg, FL).

Collection of Blood and Preparation of Washed Platelet Suspensions. Blood was collected from normal human volunteers who reported to be without medication for at least 10 days. Acid/citrate/dextrose solution (32) was used as an anticoagulant in a ratio of 1:6 (vol/vol). Citrated blood was centrifuged at 120 × g for 15 min at room temperature. The supernatant, platelet-rich plasma, was centrifuged at 1100 × g for 15 min and the platelet pellet was resuspended in modified Tyrode’s solution without calcium, pH 6.5 (33). Two percent EGTA was added (1:9, vol/vol) to platelet suspensions just before centrifugation at 1100 × g for 10 min, and this washing procedure was repeated three times. Platelets were finally resuspended in modified Tyrode’s solution, pH 7.4 (33) and counted in a Coulter Counter model ZF (Coulter). The platelet count was adjusted to 3 × 10^10 per ml.

Abbreviations: AFC, N-acetyl-S-trans,trans-farnesyl-L-cysteine; AGC, N-acetyl-S-trans,trans-geranyl-L-cysteine; [Ca2+]i, cytosolic calcium ion concentration; DAG, diacylglycerol; fura 2/AM, 1,2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl-2-(2-amin-5'-methylenephenoxy)ethane-N,N,N',N'-tetraacetic acid, pentacacetoxymethyl ester; GTP[yS], guanosine 5′-[γ-thio]triphosphate; IP, inositol 1,4,5-triphosphate; PDBu, phorbol 12,13-dibutyrate; PLC, phospholipase C; TXA2, thromboxane A2.

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for aggregation studies and to $1.5 \times 10^8$ per ml for measurement of cytosolic calcium ion concentration ($[Ca^{2+}]_i$).

**Platelet Aggregation and Serotonin Secretion Assays.** Platelet aggregation studies were conducted according to the turbidometric method of Born (34), as modified by Mustard et al. (35). A Payton dual-channel aggregation module 600B, interfaced with an Apple IIe microcomputer (36), was used to perform the aggregation studies. The secretion of platelet contents from dense granules was measured by monitoring the secretion of radioactivity from platelets prelabeled with $[^{14}C]serotonin$ (32). Washed platelets were incubated with $[^{14}C]serotonin$ (0.5 μCi per 3 × 10^11 platelets) for 20 min, washed twice, and then resuspended in modified Tyrode's solution, pH 7.4. The secretion of $[^{14}C]serotonin$ from platelets was determined by centrifuging the samples at 12,000 × g for 2 min in an Eppendorf centrifuge and assaying a 100-μl aliquot of the supernatant by liquid scintillation spectrometry. The secretion data were calculated as the percentage of radioactivity in platelets. The effect of AFC is expressed as the percentage of the maximum secretable radioactivity induced by a given agonist.

**Measurement of Platelet $[Ca^{2+}]_i$.** Platelet $[Ca^{2+}]_i$ was quantified by using fura 2/AM-loaded platelets. Washed platelets were incubated with 5 μM fura 2/AM at room temperature for 30 min. After incubation, platelets were washed twice and resuspended in a modified Tyrode's solution, pH 7.4. Fluorescence was recorded with a Perkin–Elmer LS5B fluorescence spectrophotometer (excitation, 340 nm; emission, 500 nm). $[Ca^{2+}]_i$ was estimated by using the following equation: $[Ca^{2+}]_i = K_d[(F - F_{min})/(F_{max} - F)]$, where $K_d$ was the apparent dissociation constant of fura 2, $F_{max}$ (maximum fluorescence) and $F_{min}$ (minimum fluorescence) were determined according to Tsien et al. (37). $F_{max}$ was recorded in Triton X-100-lysed samples in the presence of 3 mM calcium. The $F_{min}$ value was obtained by measuring fluorescence in the presence of 8 mM EGTA, pH 8.5.

**RESULTS**

AFC Inhibits Agonist-Receptor-Mediated Stimulus-Response Coupling. ADP, collagen, U46619, and thrombin all induce platelet aggregation by interacting with specific receptors, whereas arachidonic acid induces platelet aggregation by being converted into prostaglandins, specifically into thromboxane A2 (TXA2) (38–43). AFC (1–10 μM) inhibited platelet aggregation induced by ADP, collagen, arachidonic acid, and U46619 (Fig. 1). Slightly greater concentrations of AFC (10–50 μM) were needed to inhibit thrombin-induced platelet aggregation (Fig. 1). AFC also inhibited both thrombin- and U46619-induced serotonin secretion (Fig. 2). Furthermore, AFC blocked U46619-induced platelet aggregation and serotonin secretion responses in the presence or absence of aspirin (0.5 mM).

AFC Inhibits G-Protein-Mediated Stimulus-Response Coupling. G proteins undergo a carboxyl methylation event that is dramatically stimulated by GTPγS and inhibited by AFC (9, 28, 44). Thus, agonist-induced GTP binding was expected to induce an increase in G-protein methylation. If such methylation reaction(s) are involved in agonist-receptor-G-protein-mediated signaling pathway, then inhibition of methylation by AFC would block G-protein-mediated platelet activation. This possibility was examined by determining the effects of AFC on GTPγS-induced platelet aggregation. GTPγS induced platelet aggregation in saponin-permeabilized washed human platelets (Fig. 3). AFC inhibited GTPγS-induced platelet aggregation in a concentration-dependent manner (Fig. 3). In control experiments, GTPγS-induced platelet aggregation was completely blocked by guanosine 5'-[β-thio]diphosphate (data not shown).

**FIG. 1.** Effects of AFC on platelet aggregation induced by ADP, collagen, arachidonic acid, U46619, or thrombin. Washed human platelet samples were incubated with AFC (concentration shown beside each tracing) for 1 min prior to addition of ADP, collagen (coll), arachidonic acid (AA), U46619, or thrombin (Thr) at the indicated concentrations. The tracings are representative of four or five experiments.

AFC Is a Poor Inhibitor of Platelet Aggregation. The effects of AGC, which has been shown to be a poor inhibitor of protein methylation (9), on agonist-induced platelet aggregation were examined to determine correlation, if any, between inhibition of protein methylation and blockade of platelet

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**Pharmacology: Huzoor-Akbar et al.**

aggregation responses. AGC at concentrations up to 100 μM produced little inhibitory effect on U46619-induced platelet aggregation (Fig. 4).

**AFC Does Not Inhibit Agonist-Receptor-Independent Stimulus-Response Coupling.** Ionomycin, PDBu, and bacterial PLC induce platelet aggregation by mechanisms that do not involve receptor-mediated signal transduction. Ionomycin induces platelet aggregation by increasing [Ca\(^{2+}\)], and PDBu and PLC, by mimicking or generating DAG. The effects of AFC on platelet aggregation induced by these agents are shown in Fig. 5. Concentrations of AFC which completely inhibited agonist-receptor-mediated responses did not inhibit platelet aggregation induced by ionomycin, PLC, or PDBu. Staurosporine, a selective inhibitor of protein kinase C, completely inhibited platelet aggregation induced by PDBu (Fig. 5) and PLC (data not shown).

**AFC Inhibits Agonist-Receptor-Mediated But Not Ionomycin-Induced Increase in [Ca\(^{2+}\)].** Agonist-receptor-mediated signal transduction in platelets leads to an increase in [Ca\(^{2+}\)]. The possibility that AFC inhibits platelet aggregation by inhibiting signal transduction and thereby preventing the agonist-receptor-mediated rise in [Ca\(^{2+}\)], was examined. U46619, thrombin, and ionomycin increased basal (65 ± 5 nM) platelet [Ca\(^{2+}\)] (Fig. 6). AFC inhibited the increases in [Ca\(^{2+}\)] induced by low (0.1 μM) (Fig. 6A) as well as high (1.0 μM) (Fig. 6B) concentrations of U46619. Slightly higher concentrations of AFC were required to inhibit the thrombin-induced increase in platelet [Ca\(^{2+}\)] (Fig. 6C). However, AFC did not inhibit ionomycin-induced increase in platelet [Ca\(^{2+}\)] from extracellular (in the presence of 2 mM calcium in the medium; Fig. 6D) or intracellular (in the presence of 1 mM EGTA in the medium; Fig. 6E) sources.

**DISCUSSION**

Agonists stimulate platelets by various mechanisms. Thrombin and U46619 interact with specific receptors that are coupled by G proteins to signal-transduction pathways. These lead to the hydrolysis of membrane phospholipids and to the generation of inositol 1,4,5-trisphosphate (IP\(_3\)), DAG, and TXA\(_2\), with subsequent mobilization of cytosolic Ca\(^{2+}\) and activation of protein kinase C (38-41). On the other hand, agents such as PDBu, PLC, and ionomycin bypass receptors and G proteins. PLC induces hydrolysis of membrane phospholipids to produce DAG, which in turn activates protein kinase C (45-47). PDBu directly activates protein kinase C. Ionomycin directly mobilizes Ca\(^{2+}\) from intracellular sources. If AFC affects platelet activation by inhibiting the methylation of γ subunits of G proteins or low molecular weight GTP-binding proteins, such as rap1, it would be expected to inhibit platelet activation by agonists such as thrombin or U46619, but not agents such as PDBu, PLC, or ionomycin.

AFC completely inhibited platelet aggregation induced by thrombin and collagen (Fig. 1). Thrombin and collagen induce platelet aggregation by multiple mechanisms, including secretion of ADP and production of TXA\(_2\), IP\(_3\), and DAG (42). ADP induces a characteristic biphasic aggregation response in human platelets. The secondary phase of ADP-induced platelet aggregation, which is mediated by prostaglandins synthesized during primary aggregation, was inhibited by AFC (Fig. 1). AFC also inhibited arachidonic acid-induced platelet aggregation. Arachidonic acid induces platelet aggregation by being converted into prostaglandins (43). Our results suggest that AFC inhibits platelet aggregation by inhibiting the synthesis or the action of prostaglandins, or both. U46619, a stable analog of prostaglandin H\(_2\), induces platelet aggregation by directly interacting with TXA\(_2\) receptors (38). AFC inhibited U46619-induced aggregation and serotonin secretion in the presence or absence of aspirin (0.5
Fig. 5. Effects of AFC on platelet aggregation induced by ionomycin, PDBu, or PLC. Washed human platelet samples were incubated with AFC (concentration shown beside each tracing) for 1 min prior to addition of the indicated concentrations of ionomycin, PDBu, or bacterial PLC. The tracings are representative of three to five experiments.

Fig. 6. Effects of AFC on increase of platelet [Ca2+]i induced by U46619, thrombin, or ionomycin. Fura 2/AM-loaded washed human platelet samples were incubated with AFC for 1 min prior to addition of U46619, thrombin, or ionomycin. (A) Platelets were stimulated with U46619 (0.1 μM) in the presence of AFC (0; 3; 10; 30 μM). (B) Platelets were stimulated with U46619 (1.0 μM) in the presence of AFC (0; 3; 10; 30 μM). (C) Platelets were stimulated with thrombin (0.003 unit/ml) in the presence of AFC (0; 3; 10; 30 μM). (D) Platelets, in a calcium-rich (2.0 mM) medium, were stimulated with ionomycin (45 nM) in the presence of AFC (0; 30 μM). (E) Platelets, in a calcium-poor (1.0 mM EGTA) medium, were stimulated with ionomycin (1 μM) in the presence of AFC (0; 30 μM). The data shown are representative of four or five experiments.

mM). These findings clearly indicate that AFC blocks the action of TXA2.

Inhibition of receptor-dependent G-protein activation by thrombin, U46619, or TXA2 would inhibit stimulation of inositol phospholipid-specific PLC and consequently the production of IP3 and DAG. IP3 induces platelet aggregation by mobilizing Ca2+ from the dense tubular system. AFC inhibited thrombin- and U46619-induced, but not ionomycin-induced, increases in [Ca2+]i (Fig. 6). Thus, AFC inhibits agonist-receptor-mediated, but not direct, Ca2+ mobilization.

A number of antiplatelet agents such as prostaglandin E1, prostacyclin, and methylxanthines inhibit platelet aggregation by increasing platelet cAMP levels (41, 48–50). Increase in cAMP inhibits platelet activation mediated by diverse stimuli including thrombin and TXA2 as well as PDBu and PLC. The possibility that AFC may also be inhibiting platelet activation by increasing cAMP was examined. AFC did not increase or decrease the basal cAMP (4.1 pmol per 1 × 10^9 platelets) levels (data not shown). This finding demonstrates that AFC does not inhibit platelet activation by elevating cAMP level. In this regard, AFC appears to be different from other methylation inhibitors such as 3-deazaadenosine and L-homocysteine thiolactone. We have shown earlier that...
3-deazaadenosine and l-homocysteine thiolactone increase platelet CaMP and inhibit platelet aggregation induced by receptor-dependent (U46619) as well as receptor-independent (PLC) mechanisms (50). Thus the antiplatelet actions of 3-deazaadenosine and l-homocysteine thiolactone, but not those of AIC, may be attributable to an increase in CaMP level.

DAG induces aggregation by stimulating a protein kinase C-dependent phosphorylation of a 47-kDa protein (40). AFC does not interfere with this process, since it did not inhibit aggregation in response to PDBu (Fig. 5), an agent known to activate protein kinase C by mimicking the action of DAG (46). In addition, AFC did not inhibit PLC-stimulated aggregation (Fig. 5), a process that depends on the in situ generation of DAG (47). AFC also did not inhibit platelet aggregation induced by ionomycin (Fig. 5). These results support the conclusion that AFC inhibits platelet activation by blocking agonist-receptor-mediated signal-transduction pathways that lead to the generation of second messengers, such as IP3, Ca2+, and DAG.

The findings that AFC inhibited only agonist-receptor-induced platelet activation suggests that it may be blocking the G-protein-mediated signal transduction. This possibility is further supported by the fact that AFC also inhibited GTPγS-induced platelet aggregation (Fig. 3). Finally, it is tempting to speculate that GTPγS-dependent carboxyl methylation of rap proteins (28) may be involved in agonist-receptor-mediated signal transduction and that AFC inhibits agonist-receptor-mediated signal transduction in human platelets by blocking methylation of these proteins. A possible link between inhibition of protein methylation and inhibition of platelet aggregation is also supported by observations that AFC, which is a potent inhibitor of protein methylation, but not AICG, which is a poor inhibitor of protein methylation, inhibits agonist-receptor-mediated platelet responses.